

STRUCTURAL DIFFERENTIATION OF STACKED AND UNSTACKED CHLOROPLAST MEMBRANES

Freeze-Etch Electron Microscopy of Wild-Type and Mutant Strains of *Chlamydomonas*

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ABSTRACT

Wild-type chloroplast membranes from *Chlamydomonas reinhardtii* exhibit four faces in freeze-etch replicas: the complementary Bs and Cs faces are found where the membranes are stacked together; the complementary Bu and Cu faces are found in unstacked membranes. The Bs face carries a dense population of regularly spaced particles containing the large, 160 ± 10 Å particles that appear to be unique to chloroplast membranes. Under certain growth conditions, membrane stacking does not occur in the *ac-5* strain. When isolated, these membranes remain unstacked, exhibit only Bu and Cu faces, and retain the ability to carry out normal photosynthesis. Membrane stacking is also absent in the *ac-31* strain, and, when isolated in a low-salt medium, these membranes remain unstacked and exhibit only Bu and Cu faces. When isolated in a high-salt medium, however, they stack normally, and Bs and Cs faces are produced by this *in vitro* stacking process. We conclude that certain particle distributions in the chloroplast membrane are created as a consequence of the stacking process, and that the ability of membranes to stack can be modified both by gene mutation and by the ionic environment in which the membranes are found.

INTRODUCTION

The structural organization of chloroplast membranes in green algae and in higher plants is distinct from that of all other membranes. In fixed and sectioned material an individual chloroplast membrane does not appear unusual, being approximately 70 Å wide and exhibiting a trilaminar structure. However, in preparations of whole chloroplast membranes that are either shadowed or negatively stained, unique arrays of particles are seen (3, 28, 40, 42). Moreover, when chloroplast membranes are freeze-cleaved or freeze-etched, faces are revealed that exhibit not only a

higher particle density than other membranes (8), but also a distinctive type of large particle found in no other membrane that has yet been studied. These large particles have been termed "quintasomes" or, more recently, "quintasome cores" by Park and coworkers (9, 42, 43) who have suggested that the particles might be the morphological counterpart of some functional unit of photosynthesis (41, 44).

Chloroplast membranes also differ from most membranes in that they possess the capacity to fuse together over long distances, forming "grana" in

higher plants and smaller "stacks" in green algae. Membrane fusion occurs in myelin (47) and in certain specialized regions of the plasma membrane such as tight junctions (16, 20, 49); however, most membranes do not make contact over extensive distances under physiological conditions.

The two distinctive structural properties of chloroplast membranes—their particle population and their ability to fuse—are the subject of the present investigation. We have studied the appearance of chloroplast membranes from three different strains of the unicellular green alga, *Chlamydomonas reinhardtii*, by electron microscopy of thin-sectioned and freeze-etched material. Freeze-etched replicas from wild-type membranes exhibit the same types of faces and particles as do replicas of membranes from higher plant chloroplasts. However, our interpretation of such replicas differs in certain important respects from those that have been offered by other investigators. We find that stacked membranes possess a freeze-etch morphology that is distinct from that of unstacked membranes. Parallel studies of two mutant strains, *ac-5* and *ac-31*, strongly support this conclusion, for under certain growth or experimental conditions, these strains possess exclusively unstacked membranes and exhibit only the morphology characteristic of unstacked membranes in freeze-etched preparations.

A comparison of the membrane structure of these strains has enabled us to draw the following conclusions: (a) the characteristic arrays of large particles found within chloroplast membranes are produced when stacking occurs and are absent from unstacked membranes, whether or not these membranes possess the potential for stacking; (b) a dramatic reduction in the number of large particles is not accompanied by any loss of photosynthetic capacity as measured by CO₂ fixation or photosynthetic electron-transport reactions at high light intensities; (c) chloroplast membrane stacking, and the concomitant formation of distinct membrane faces, is mediated by ionic interactions between membranes; (d) the chloroplast membranes appear to be differentiated into regions that can and cannot stack.

MATERIALS AND METHODS

Culture of the Organisms

Wild-type (strain 137c) and mutant (strains *ac-5* and *ac-31*) cells were grown in 3-l volumes of minimal

medium (50) or minimal medium supplemented with 3% sodium acetate contained in 4-l Erlenmeyer flasks and agitated with magnetic stirrers. The light intensity from daylight fluorescent lamps was 2000 lux, and the temperature was maintained at 26°C. Cultures were harvested in the logarithmic phase of growth.

Preparation of Chloroplast Fragments

A sample of intact cells was first fixed for thin-section electron microscopy by methods previously described (23, 31). The remaining cells were washed and broken gently in a French pressure cell (35–50 kg/cm²). The broken preparation was subjected to differential centrifugation as described by Surzycki (51) to eliminate whole cells, starch, and small membrane fragments, but no attempt was made to isolate whole chloroplasts; the final "chloroplast fraction" therefore contained some protoplasts and chloroplasts and a predominance of large chloroplast membrane fragments.

All procedures were carried out at 4°C. In most cases the buffers used were those described by Surzycki (51): for washing, 0.05 M Tris HCl (pH 8), 0.25 M sucrose, 0.001 M Mg EDTA, and 0.02 M MgSO₄; for breaking and subsequent centrifugations, 0.05 M Tris HCl (pH 8), 0.5 M sucrose, 0.02 M MgSO₄, and 0.25% bovine serum albumin. This second solution will be referred to in the text as high-salt buffer. To isolate unstacked *ac-31* chloroplast membranes, cells were washed, broken, and centrifuged in 0.05 M Tricine NaOH, pH 7.3 (19); this will be referred to as low-salt Tricine buffer. To prepare unstacked wild-type membranes, cells were washed, broken, and centrifuged as usual in high-salt buffer, and the fragments were then subjected to five washings in the low-salt Tricine buffer over a 1 hr period.

The final chloroplast membrane fraction was suspended in 11 ml of cold high- or low-salt buffer, and 4 ml of glycerol were slowly added over a 30 min period. After an additional 30 min at the final glycerol concentration (ca. 27%), the membranes were spun down at 5000 g for 3 min. A portion of the pellet was used for preparing freeze-etch specimens; these were rapidly frozen in Freon 12 held at –150°C. The remainder of the pellet was used for thin-section microscopy. The fragments were usually washed once in 0.15 M potassium phosphate buffer, pH 7.4, containing 0.5 M sucrose and 27% glycerol. This step removes Tris, which is an unsuitable vehicle for glutaraldehyde fixation (13). The fragments were then resuspended in this solution, and an equal volume of 4% glutaraldehyde in the same buffer was added. After a 2–3 hr fixation, they were washed in the buffer without glycerol and postfixed in 1% OsO₄ in the same buffer for 45 min. They were then washed in the phosphate buffer without

sucrose or glycerol, dehydrated rapidly in ethanol, and introduced into Epon-Araldite (31) without intervening propylene oxide. They were transferred to fresh monomer after 2 days, subjected to evacuation at 80°C, and embedded at 80°C for 3 days.

To fix the low-salt preparation of *ac-31* fragments, the final pellet was suspended in the Tricine buffer containing 27% glycerol to which an equal volume of 0.004 M potassium phosphate buffer, pH 7, containing 4% glutaraldehyde was added. The remaining procedures were performed in the dilute phosphate buffer. The unstacked wild-type fragments were suspended in the Tricine buffer, and a commercial 20% stock solution of Taab (Reading, England) glutaraldehyde was added to a final concentration of 2%. The remaining procedures were performed in the dilute phosphate buffer. Both procedures for fixing in the presence of Tricine give unsatisfactory thin-section images as discussed in the text.

The freeze-etch replicas were prepared at -100°C according to the method described by Moor and Mühlenthaler (38). However, to avoid postcleaving changes of the exposed membrane surfaces, the specimens were replicated within 3-5 sec of the last pass of the microtome knife.

Thin sections were examined with a Hitachi HU-11C and replicas were examined with a Phillips 300 electron microscope.

Measurement of Photosynthetic Parameters

Total chlorophyll and chlorophyll a:b ratios were estimated by a modification (2) of the method of Mackinney (37). Cells were counted in a hemacytometer. Carbon dioxide fixation was measured as the amount of ¹⁴CO₂ fixed by whole cells, by using a method described elsewhere (21). The activity of PS II¹ was measured as the light-induced reduction of DPIP from water, and PS I activity was measured as the light-induced reduction of NADP from reduced DPIP. The total operation of the photosynthetic electron-transport chain was measured as the light-induced reduction of NADP from water. Assays were made using chloroplast fragments prepared by the sonic disruption of cells. References to methods will be found in reference 21.

Measurement of Particle Sizes

Particle sizes were determined from micrographs enlarged to 160,000 × and viewed through an 8 × objective lens equipped with a micrometer grating. The width of the shadow of a given particle was

¹ Abbreviations used: PS II, photochemical system II; PS I, photochemical system I; DPIP, 2,6-dichlorophenylindophenol; NADP, nicotinamide adenine dinucleotide phosphate.

measured over the shadowed half of the particle. Where the shadow boundary appeared fuzzy or irregular, a minimum width value was always taken. Between 200 and 400 particles were measured in large continuous areas on at least two different micrographs for each histogram of particle size distribution (Fig. 9). Histograms of "B"-type faces are more easily obtained and are therefore used to define changes in particle populations of freeze-etched membranes.

RESULTS

Wild-Type Chloroplast Membranes

The organization of chloroplast membranes in whole cells of wild-type *C. reinhardi* is shown in Fig. 1. Stacks of 2-10 thylakoids, or discs, lie within the chloroplast stroma, as described in detail elsewhere (22). The apposed membranes in a stack apparently make direct contact, for the width of two stacked membranes is consistently twice that of a single, unstacked membrane in sectioned material (22).

C. reinhardi is impermeable to glycerol, and attempts to produce freeze-etch replicas from unglycerinated cells were unsatisfactory. We therefore isolated large chloroplast fragments and suspended them in a buffered 27% glycerol solution. Thin-section microscopy of such fragments (Fig. 2) reveals that the chloroplast thylakoids retain their stacked configuration even after disruption, extensive washing, and chemical fixation, as has also been observed for preparations of higher plant chloroplast fragments (32, 56). In other words, the association between chloroplast membranes appears to be a very strong and durable one.

Fig. 3 shows at low magnification, a typical freeze-etched replica obtained from unfixed wild-type chloroplast membranes, and Figs. 4-8 show the characteristic membrane faces of such replicas at higher magnifications. Experimental results of others (43, 45, 53, 55) have supported the theory (6, 9) that the fracture plane travels preferentially along the interior of the membrane in such a way that two complementary faces are exposed. As this theory is consistent with our observations, it forms the basis of our interpretations of freeze-etch replicas. We have called the two complementary faces of chloroplast membranes B and C, since they are equivalent to the B and C faces of Branton and Park (9). However, we find evidence for two different types of B and C faces, namely, those that occur within areas of membrane that are stacked

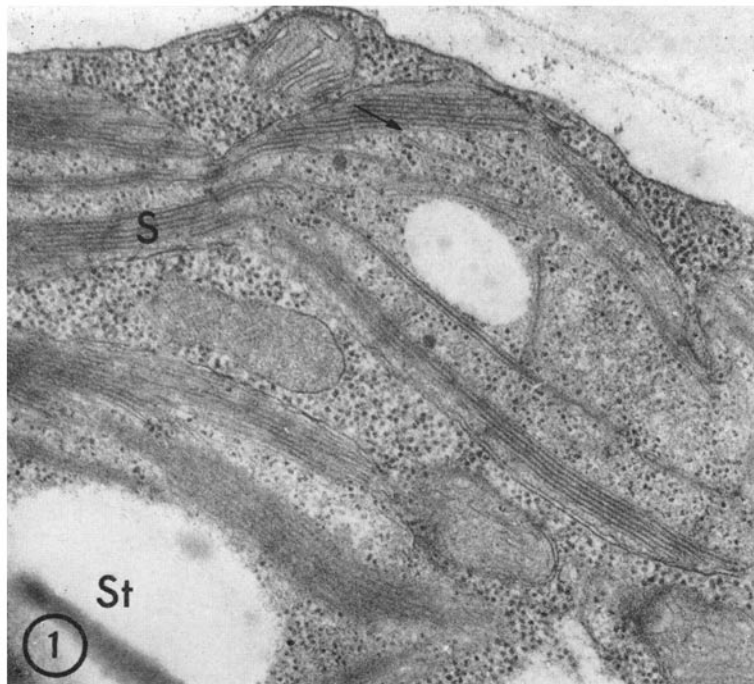


FIGURE 1 Portion of a wild-type *C. reinhardtii* cell grown mixotrophically, showing two lobes of the chloroplast. Thylakoids are usually found in stacks (*S*) of 2–7 thylakoids. *Arrow* indicates region where single thylakoid joins a larger stack. Starch is shown at *ST*. $\times 48,000$.

and those that occur within areas of membrane that are unstacked. We shall therefore refer to the faces that derive from stacked membranes as Bs and Cs, and those that occur within unstacked membranes as Bu and Cu. These faces and the particles associated with them are described below.

The Bs Face

The Bs is the most distinctive face of chloroplast membranes and corresponds to the B face of Branton and Park (see Figs. 5, 6, and 9 of reference 9). It is characterized by a dense population of fairly regularly spaced particles. We have measured the size of more than 400 of these particles, and the results are plotted in Fig. 9 *a*. It is evident from this histogram, as well as from a careful inspection of Figs. 3 and 5, that the particles are not uniform in size. The majority of the particles seem to fall into three major size categories: 105 ± 10 Å, 130 ± 10 Å, and 160 ± 10 Å. It is most likely that our 160 ± 10 Å particle corresponds to the 175 Å “quintasome” of Branton and Park (9). It often

appears to be composed of subunits, although we should point out that the smaller particles on the Bs face also occasionally appear to be made up of subunits. Particles in the 160 ± 10 Å category account for some 22% of the particles on the wild-type Bs membrane face (Fig. 9 *a*). We cannot exclude the possibility that at least some of the smaller particles found on this face might arise from larger particles that are torn apart during the cleaving process, as discussed more fully in a later section.

We have chosen to characterize our membrane faces by particle-size distribution data rather than by estimates of particle density. Use of the latter parameter requires making a choice of which membrane area to count; as is evident in the figures in this paper, particle density varies considerably from one region of a given face to the next. Very marked differences in particle density, such as the difference between Bs faces and the Bu faces described below, are sufficiently obvious to render quantitation unnecessary.

The Bs face usually appears to have a very

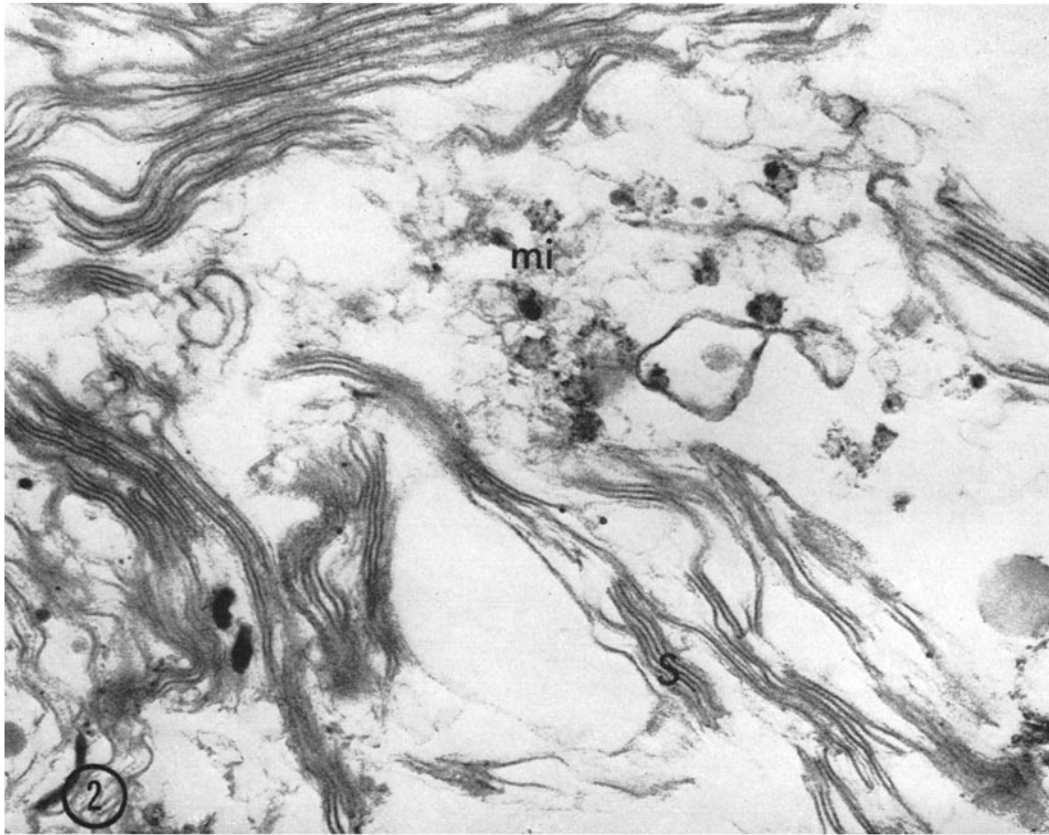


FIGURE 2 Isolated chloroplast membranes from wild-type cells, glutaraldehyde-fixed in 27% glycerol in high-salt buffer. Thylakoids retain their stacked configuration (*S*), even though the chloroplast envelope has ruptured and stroma contents have been lost. Microsomes (*mi*) and other membranes are also present in the preparation. $\times 48,000$.

smooth background matrix. At low shadowing angles, however, the matrix between the particles reveals large numbers of small, shallow depressions, most of which have diameters of 20–70 Å (Fig. 5, *arrows*).

As indicated in Fig. 3, Bs surfaces are found exclusively in areas of the replica that derive from regions of membrane stacking.

The Cs Face

The Cs membrane face (Figs. 3, 4, and 6) is—like the Bs face—only seen in regions where the thylakoid membranes are in a stacked configuration (Fig. 3). The Cs face appears to be quite uneven, largely because it contains a considerable number of large holes, 50–160 Å in diameter (Fig. 6, *arrows*), and, to a lesser extent, because it carries

a moderate number of small particles. Most of the particles range in size from 30 to 100 Å (Fig. 9 *c*). Our Cs membrane faces appear to correspond to the faces labeled *A* on the micrographs of Branton and Park (9) and Arntzen et al. (3), but they do not correspond to the surfaces labeled *A* in the diagrams of these authors (see Discussion).

The Bu Face

Bu faces are typical of thylakoid membrane regions that do not take part in the stacking process (Figs. 3, 7). They are continuous with the Bs faces (Fig. 3) and appear to share the same background matrix. In the Bu areas, however, the matrix is marked by numerous depressions (Fig. 7, *arrows*) that resemble pockmarks and appear much larger (50–120 Å in diameter) and deeper than the de-

pressions in the Bs matrix (Fig. 5, *arrows*). The Bu face also carries a relatively sparse population of particles. The average particle size is smaller than that of a Bs face (compare Fig. 9 *b* with Fig. 9 *a*). This difference is mainly due to a sharp decrease in the relative number of 160 ± 10 A particles; to a lesser extent it is also due to a decrease in the number of 130 ± 10 A particles and a proportional increase in the number of particles with diameters of 80–115 A. Notice also the shift in the average size of the smaller particles from 105 to 95 A as one moves from a Bs to a Bu face. Branton and Park (9) refer to the Bu face as a B face from which the “quantasome” particles have been torn away, leaving large holes. In Fig. 5 of reference 9, a large Bu face is present but it is not specifically distinguished from the adjacent “B” face.

The Cu Face

Cu faces are also characteristic of unstacked thylakoid membranes (Fig. 3). They are continuous with Cs membrane faces (Figs. 3, 4) in the same way that Bu and Bs faces are continuous with each other. The background matrix of the Cu face is smoother than that of the Cs face and carries only infrequent depressions (Fig. 8, *arrows*); for this reason, one can more readily visualize its population of densely packed particles (Figs. 4 and 6). The diameters of these particles are difficult to determine because of their close packing and frequently irregular form, and thus we have only attempted to determine the particle size distribution of wild-type Cu faces. The resulting histogram

(Fig. 9 *d*) indicates that the particle sizes on a Cu face do not differ significantly from those seen on a Bu face (Fig. 9 *b*), although more small particles (<80 A in diameter) are found on a Cu face and the peaks at 95, 125, and 155 A are less pronounced. Our Cu faces correspond to the C faces of Branton and Park (9) and of Arntzen et al. (3).

To confirm our identification of stacked and unstacked membrane faces, and to obtain information on the possible nature of the 160 ± 10 A particles, we have extended our study to two strains of *C. reinhardi* that carry mutations affecting the stacking properties of their chloroplast membranes.

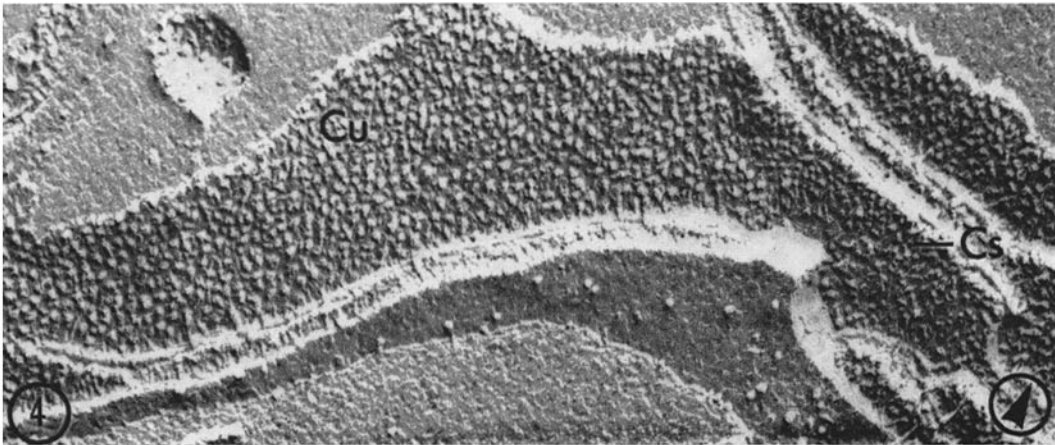
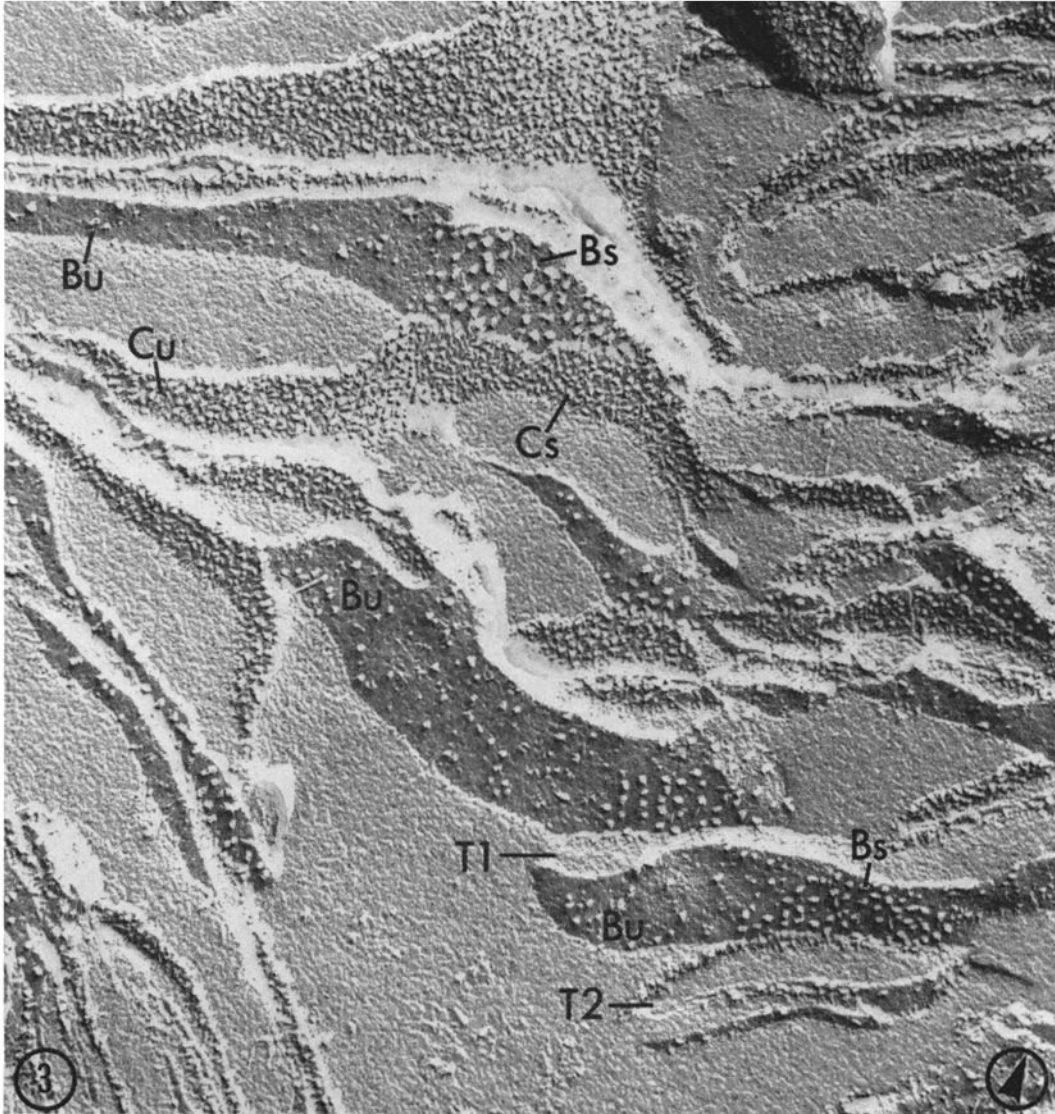
The Chloroplast Membranes of *ac-5*

The *ac-5* mutation in *C. reinhardi* produces cells that are chlorophyll deficient, and particularly chlorophyll *b* deficient, in comparison to wild-type cells (Table I). The mutant cells can be grown either phototrophically on a minimal medium or mixotrophically in the light on a minimal medium supplemented with acetate; under both growth conditions the cells are equally pigment deficient or, if anything, the phototrophic cells tend to be more pigment deficient than the mixotrophic cells (Table I). The photosynthetic capacity of *ac-5* cells is also comparable under both growth conditions, whether measured as the ability of whole cells to fix CO₂ in the light or as the ability of isolated chloroplast fragments to photoreduce DPIP or NADP (Table I). Thus, there is no apparent difference in PS I or PS II activity under the two growth conditions. It should be noted that these

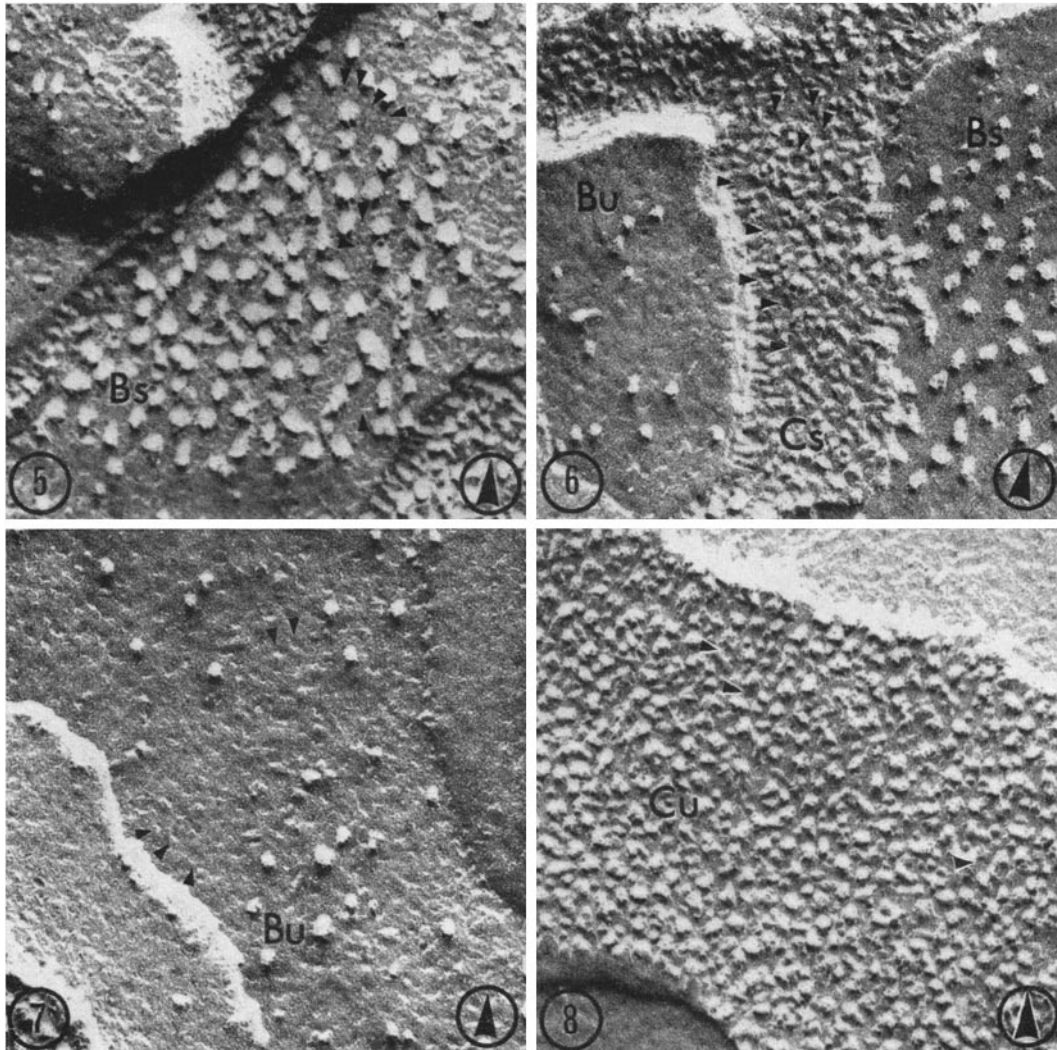
Figs. 3 and 4 on following page.

FIGURE 3 Freeze-etched isolated chloroplast membranes from wild-type cells, suspended in 27% glycerol in high-salt buffer. Four types of split inner membrane faces can be distinguished; they have been labeled *Bs*, *Bu*, *Cs*, and *Cu*, for reasons discussed in the text. Faces *Bs* and *Bu* are continuous with each other and face outwards with respect to the lumen of the thylakoids; *Cs* and *Cu* are also continuous but face inwards (See Figs. 4 and 21). *T1* and *T2* are two adjacent thylakoids. Where they come together to form a stacked region, a typical *Bs* (B stacked) face is seen. The adjoining *Bu* (B unstacked) face, which leads up to the edge of the stacked region, is characteristic of unstacked membrane areas. The relationship between *Cs* and *Bs* faces in stacked membrane regions is apparent in the upper half of the picture. The *Cs* face changes into a *Cu* face where the membranes become unstacked. Faces *Bs*, *Cs*, *Bu*, and *Cu* are shown in greater detail in Figs. 5–8. Circled triangle in lower right shows the angle of shadowing. $\times 80,000$.

FIGURE 4 Micrograph demonstrating both the continuity between *Cs* and *Cu* faces and their fine-structural differences. The *Cs* face appears as a rough, irregular surface with predominantly small particles (See Fig. 9 *c*) distributed between large depressions. The *Cu* face has a more regular appearance with numerous, medium-sized particles (Fig. 9 *d*) protruding from a relatively smooth background in which few holes can be detected. Wild-type chloroplast preparation suspended in 27% glycerol in high-salt buffer. Circled triangle in lower right shows the angle of shadowing. $\times 100,000$.



Legend for Figs. 3-4 on page before Figs.



FIGURES 5-8 The four membrane faces *Bs*, *Cs*, *Bu*, and *Cu* from wild-type chloroplast preparations suspended in 27% glycerol in high-salt buffer. The suggested relationships of these faces to one another are indicated schematically in Fig. 23 *B*, and are discussed in detail in the text. Fig. 5, A *Bs* face from a replica area with a low shadowing angle, demonstrating the fine-structural details in the background matrix. Numerous small holes (*arrows*), with diameters of 20-80 Å, interrupt the smooth matrix. A population of regularly spaced large particles (Fig. 9 *a*) is also seen; some of these exhibit a substructure. Fig. 6, A *Cs* face, exhibiting numerous large holes (*arrows*) that are distributed between the particles (Fig. 9 *c*) of the background material. On the right edge of the *Cs* face it is seen that the tips of the large particles of the underlying *Bs* face are approximately level with the tips of the particles on the *Cs* face. Some of the large *Bs* particles at the edge seem to be continuous with and even identical with, a part of the background material making up the *Cs* face. Fig. 7, A *Bu* face, which carries few heterogeneously sized particles (Fig. 9 *b*) and exhibits many large depressions (*arrows*) within its relatively smooth background matrix. Fig. 8, A *Cu* face, covered with numerous, medium-sized particles (Fig. 9 *d*); these are set in a smooth background material in which only a few holes (*arrows*) can be recognized. Circled triangle in lower right shows the angle of shadowing. Figs. 5-8 $\times 160,000$.

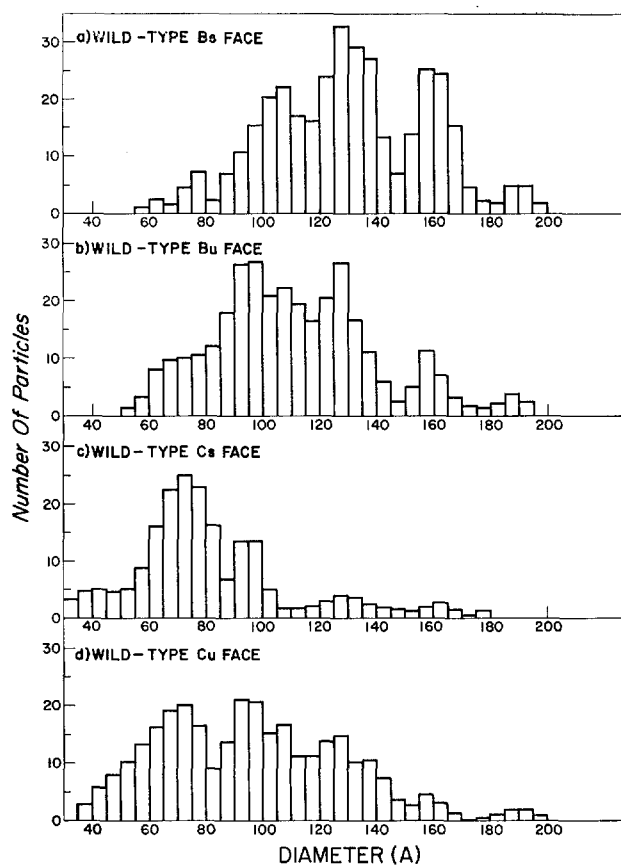


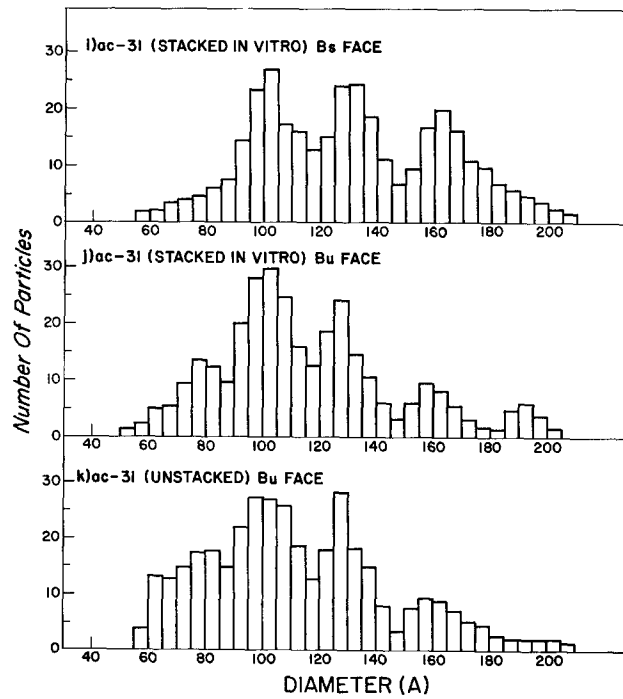
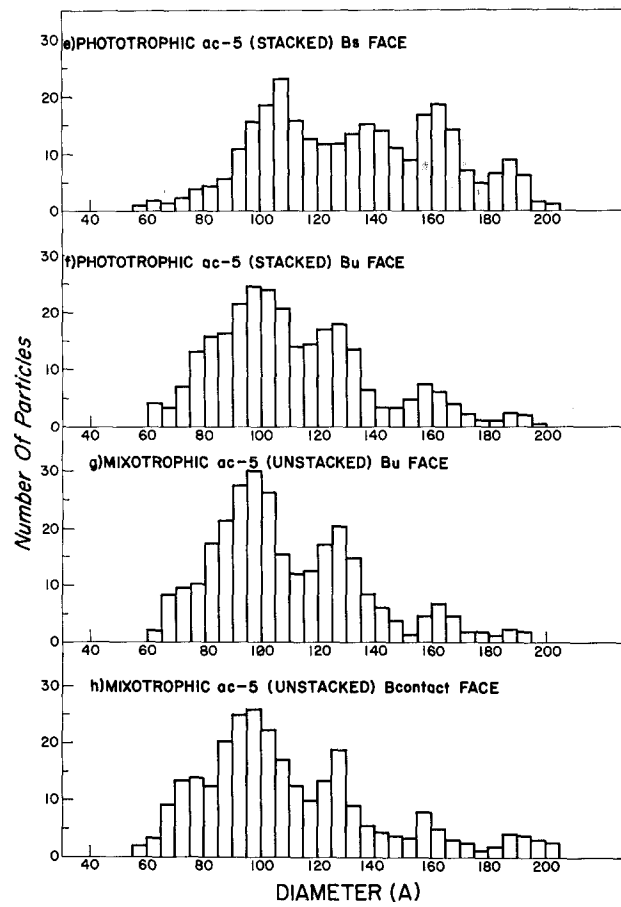
FIGURE 9 Histograms of particle sizes found on various membrane faces in freeze-etch preparations of *C. reinhardtii* chloroplast membranes. The histograms are labeled *a-k*, and each is identified by individual labels.

assays were all performed at light intensities that are saturating for wild-type cells; we do not yet have information on the photosynthesis of *ac-5* at low light intensities, although such experiments are now being carried out by R. P. Levine.

When the fine structure of *ac-5* cells is examined, a striking difference is found between phototrophically grown and mixotrophically grown cells. Figs. 10 and 11 show, respectively, a phototrophic cell and chloroplast fragments from a phototrophic cell. It is evident that most of the chloroplast stacks contain only two thylakoids and that there are many more single thylakoids present than are found in wild-type chloroplasts (compare with Figs. 1 and 2). Nonetheless, membrane stacking unquestionably occurs. In contrast, mixotrophic *ac-5* cells are incapable of any chloroplast mem-

brane stacking (Fig. 12). The membranes traverse the chloroplast stroma in an apparently independent manner, and no examples of fusion have ever been found. When chloroplast fragments are isolated from such cells, moreover, the membranes remain unstacked (Fig. 13), although, very occasionally, one encounters short regions where the membranes give the appearance of having fused (Fig. 13, *arrows*).

Freeze-etch replicas of isolated phototrophic and mixotrophic *ac-5* chloroplast membranes are shown in Figs. 14–16. Phototrophic *ac-5* replicas exhibit the four types of membrane faces, Bs, Cs, Bu, and Cu, found in wild-type replicas (Fig. 14). The histograms of particle sizes on the B faces also correspond to those of the wild type (compare Figs. 9 *e* and *f* with Figs. 9 *a* and *b*). In contrast, only



For legend see preceding page.

TABLE I

Chlorophyll (chl) Content and Rates of Photosynthetic Reactions of the Wild-Type, ac-5, and ac-31 Strains of C. Reinhardtii.

Data are taken from single, representative experiments.

Strain	Growth condition	Total chloro-phyll a:b	Chloro-phyll a:b	CO ₂ fixation		DPIP photoreduction		NADP photoreduction		NADP photoreduction from DPIP-ascorbate	
				$\mu\text{moles CO}_2$ fixed/hr per mg chloro-phyll per 10 ⁸ cells	$\mu\text{moles CO}_2$ fixed/hr per 10 ⁶ cells	$\mu\text{moles photo-re-duced/hr per mg chl}$	$\mu\text{moles photo-re-duced/hr per 10}^6$ cells	$\mu\text{moles photo-re-duced/hr per mg chl}$	$\mu\text{moles photo-re-duced/hr per 10}^6$ cells	$\mu\text{moles photo-re-duced/hr per mg chl}$	$\mu\text{moles photo-re-duced/hr per 10}^6$ cells
Wild type	Mixotrophic	3.6	2.5	152	0.551	201	0.725	101	0.367	90	0.325
	Phototrophic	1.8	2.6	152	0.279	142	0.240	102	0.172	58	0.099
<i>ac-5</i>	Mixotrophic	0.66	3.4	230	0.151	335	0.265	134	0.106	108	0.085
	Phototrophic	0.61	3.0	230	0.140	356	0.196	128	0.070	120	0.066
<i>ac-31*</i>	Mixotrophic	1.1	3.4	184	0.202	265	0.169	232	0.148	91	0.058

* Data taken from previously published experiments (21).

Bu and Cu faces are found in replicas from the unstacked, mixotrophic *ac-5* cells (Figs. 15–16). The distribution of particle sizes on the Bu faces of mixotrophic and phototrophic *ac-5* membranes is comparable (compare Fig. 9g with Fig. 9f). The Bu faces of mixotrophic *ac-5* cells tend to carry a somewhat higher density of particles than the Bu faces of wild-type cells (compare Figs. 3 and 16), but it is difficult to express this observation quantitatively since, as we have pointed out earlier, particle densities vary so markedly from one membrane area to another.

Occasionally, small regions are encountered in replicas of mixotrophic *ac-5* cells that, we believe, correspond to the short regions of membrane contact occasionally seen in thin section (Fig. 13). One such region is included in Fig. 15 (arrow): two membranes are seen to come together, and in the region of contiguity the density of particles increases on the exposed membrane face. When examined by eye, such regions appear to lack the arrays of large particles typical of truly stacked membranes, and this impression is confirmed when particle size measurements are made. As seen in Fig. 9h, the distribution of particle sizes in the regions of membrane contact remains the same as on Bu faces. No Bs particle distribution is created and, in particular, no increase is observed in the relative number of 160 ± 10 Å particles. Thus freeze-etching allows a clear distinction between

true chloroplast membrane stacking and regions of close membrane association.

The Chloroplast Membranes of *ac-31*

A detailed study of the *ac-31* strain has been published elsewhere (21). Table I summarizes the photosynthetic properties and pigment content of this strain, all of which are seen to be comparable to those of the *ac-5* strain. It should be noted that, since both strains are pigment deficient, both give rates of photosynthetic reactions that are high compared with those of the wild type when calculated on a chlorophyll basis, and low compared with those of the wild type when calculated on a cell basis. It is thus difficult to make meaningful comparisons between the photosynthetic capacities of these cells and wild-type cells, as discussed more fully elsewhere (21).

The chloroplast membranes of *ac-31* cells are unstacked in vivo (Fig. 17), although the thylakoids tend to lie much closer together than do the thylakoids of mixotrophic *ac-5* (compare with Fig. 12). The cell shown in Fig. 17 was grown mixotrophically; phototrophically grown cells of *ac-31*, unlike those of *ac-5*, do not possess stacked membranes, although the tendency for membranes to “travel together” in the stroma is even more pronounced under phototrophic conditions (21).

When we isolated chloroplast fragments from

ac-31 cells by procedures identical to those followed for wild-type and *ac-5* cells, we found, to our great surprise, that the chloroplast membranes in the fragments were stacked. This is shown in Fig. 18. The in vitro stacking, moreover, does not appear to be the result of some random aggregation; rather, the thylakoids form anastomosing stacks of 2-4 thylakoids that are indistinguishable from wild-type stacks (compare Figs. 2 and 18).

In an attempt to prevent the in vitro stacking process, and thus to visualize *ac-31* membranes in their unstacked state, we tried adding glycerol slowly to a suspension of whole cells in acetate medium. After 2 hr when the glycerol concentration in the medium had reached 10%, the cells had shrunk to small compact masses; water was clearly leaving the cells in response to the presence of glycerol in the medium. When examined in thin section, these shrunken cells proved to possess stacked membranes, leading us to suspect that an increase in the ionic environment of the *ac-31* chloroplast membranes, whether brought about by removing water from whole cells or by exposing isolated fragments to high-salt buffers, might permit the membranes to stack.

On this premise we isolated *ac-31* chloroplast fragments in 0.05 M Tricine, a zwitterion that buffers effectively without introducing ions into the medium (19). The resulting preparation was found to be completely unstacked in thin section (Fig. 19); regions of even transitory contact could not be found.² The membranes have a somewhat fragmented appearance in section and stain poorly; we suspect that this reflects a poor fixation produced by the presence of Tricine in the glutaraldehyde fixation medium (13).

Freeze-etch preparations of *ac-31* membranes that have undergone in vitro stacking exhibit the four membrane faces found in wild-type and in phototrophic *ac-5* cells (Fig. 20), and these faces carry the usual distribution of particles (Figs. 9 *i* and 9 *j*). In particular, the Bs faces of in vitro

stacked membranes are indistinguishable from those found within normally stacked membranes (compare Figs. 20 and 3), and they carry a substantial proportion of 160 ± 10 Å particles (Fig. 9 *i*). In contrast, *ac-31* membranes that are isolated in their unstacked configuration possess only Bu and Cu faces (Fig. 21). As with the mixotrophic *ac-5* chloroplast membranes, the particle sizes on the Bu faces of this material are comparable to those found on wild-type Bu faces (compare Figs. 9 *k* and 9 *b*). Again, the particle density appears to be somewhat higher in the unstacked mutant membranes.

Unstacked Wild-Type Chloroplast Membranes

Our experience with *ac-31* indicated that membrane stacking in *C. reinhardi* is mediated by ionic interactions. To explore this point further, we isolated wild-type chloroplast fragments by our usual procedure and then washed them extensively with low-salt Tricine. As Izawa and Good had earlier found in studies of isolated spinach chloroplasts (29), such treatment leads to an almost complete unstacking of the wild-type membranes; only occasional regions of fusion remain. A freeze-etch replica of such unstacked wild-type membranes is shown in Fig. 22: only Bu and Cu membrane faces are present (compare with Fig. 3). As with the unstacked *ac-5* and *ac-31* preparations, the particle density on these Bu faces seems to be higher than that on the Bu faces found in predominantly stacked membrane preparations. Whether this difference reflects some reduction in damage suffered by the membranes during the fracturing process, or some alteration in the fracturing properties of the membranes themselves cannot be determined from this study.

DISCUSSION

Chloroplast Membrane Faces

When biological membranes, including chloroplast membranes, are cleaved in the frozen state, the fracture plane appears to travel preferentially along the interior of the membrane in such a way that two complementary faces are exposed (6, 7, 9, 43, 45, 53, 55). In our study of the chloroplast membranes of *C. reinhardi* we have termed these two complementary faces B and C after the notation of Branton and Park (9). However, we have presented evidence to show that both faces differ

² The in vitro photosynthetic reaction rates previously published for *ac-31* (21) were undoubtedly obtained with stacked preparations; we were unaware of the in vitro stacking phenomenon at the time of this earlier study. These experiments are presently being repeated by R. P. Levine with *ac-31* fragments isolated in low-salt Tricine. The conclusions of the earlier study are not, however, discounted by the present finding, since they were based on in vivo as well as in vitro experiments.

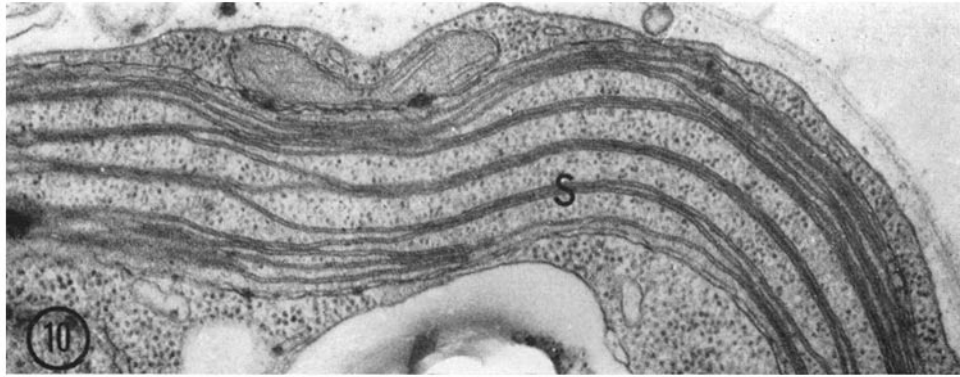


FIGURE 10 Portion of an *ac-5* cell grown phototrophically. Stacks (*S*) are primarily composed of two thylakoids each; many single discs are also present. $\times 48,000$.



FIGURE 11 Isolated chloroplast membranes from phototrophically grown *ac-5* cells, glutaraldehyde-fixed in 27% glycerol in high salt buffer. Stacks (*S*) are composed primarily of two thylakoids each. Osmiophilic globules (*G*), a swollen mitochondrion (*M*), and a dictyosome (*D*) are also present. $\times 48,000$.

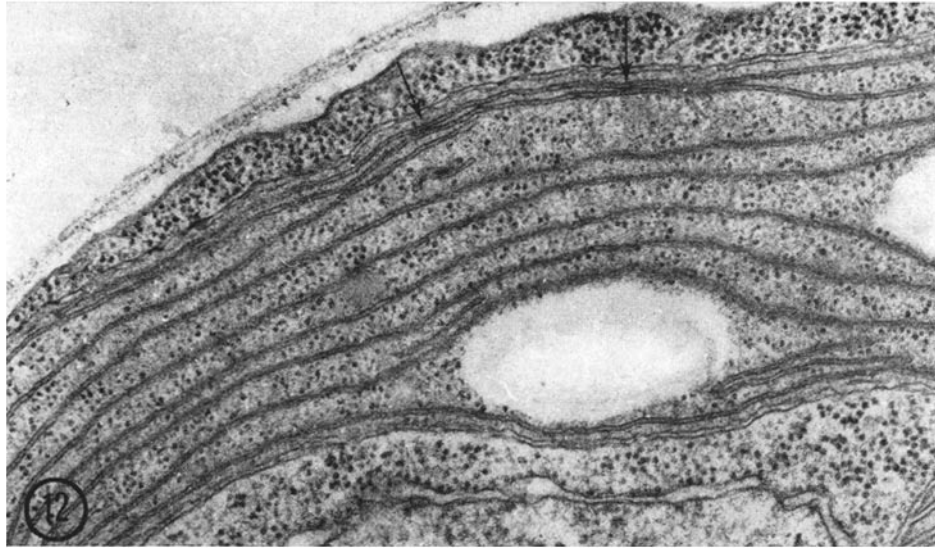


FIGURE 12 Portion of an *ac-5* cell grown mixotrophically. Single discs run an independent course through the chloroplast stroma. Arrows indicate limited sites where membranes approach one another, but fusion does not occur in such regions. $\times 48,000$.

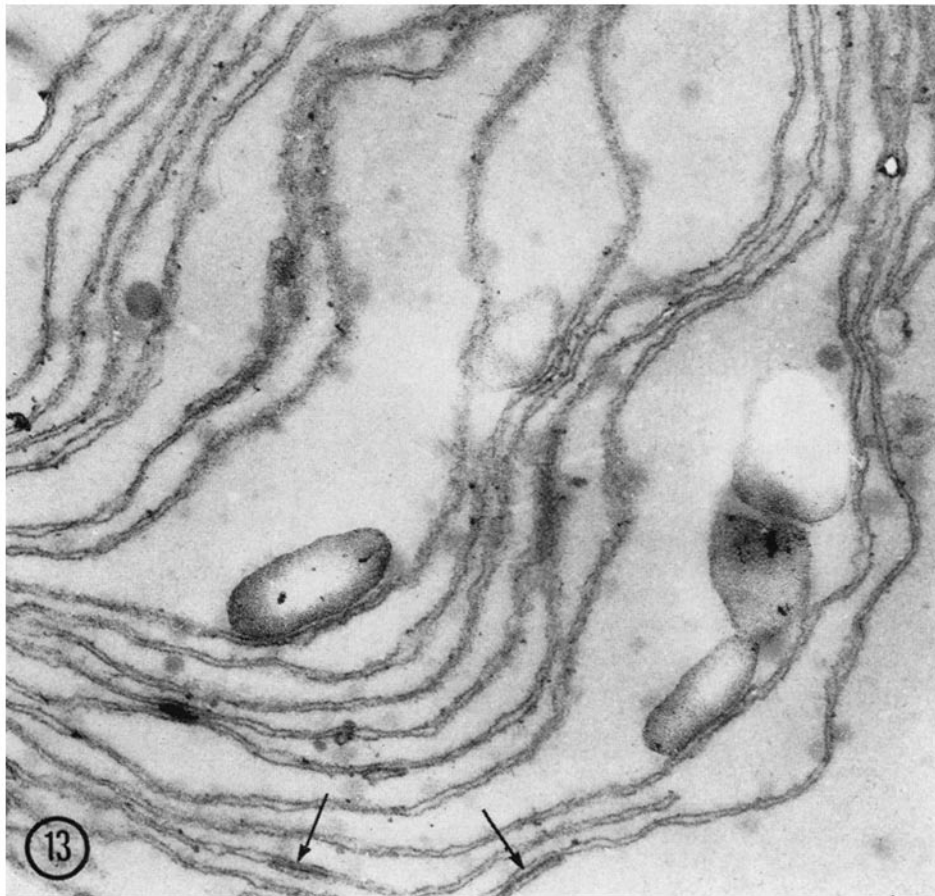


FIGURE 13 Isolated chloroplast membranes from mixotrophically grown *ac-5* cells, glutaraldehyde-fixed in 27% glycerol in high-salt buffer. Thylakoids retain their unstacked configuration. Occasional examples of membrane contact are indicated at arrows; as shown in Fig. 15, however, this contact does not result in a true membrane fusion. $\times 48,000$.

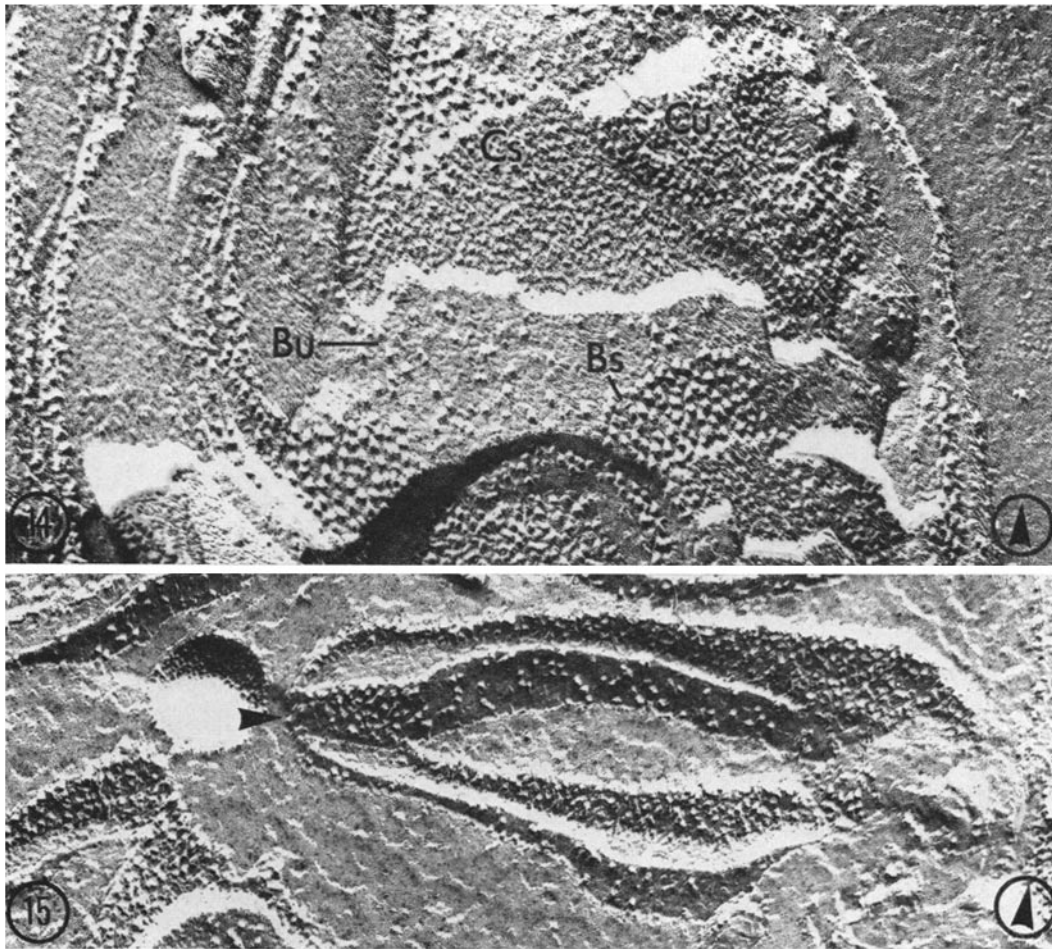


FIGURE 14 Freeze-etched isolated chloroplast membranes from phototrophically grown *ac-5* cells, suspended in 27% glycerol in high-salt buffer. The four faces of chloroplast membranes are indicated, and the Bs faces typical of stacked membrane regions can be recognized. The coarse shadowing of this replica covers up some of the finer details of the exposed membrane faces. Circled triangles in lower right of Figs. 14-16 indicate the angles of shadowing. $\times 80,000$.

FIGURE 15 Freeze-etched, isolated chloroplast membranes from mixotrophically grown *ac-5* cells, suspended in 27% glycerol in high-salt buffer. The arrow indicates a "B contact" region in which two thylakoids seem to be adhering together. Such a region is readily distinguished from truly stacked regions in that it apparently carries the same complement of particles as do Bu faces, and does not carry the ordered arrays of large particles found on Bs faces. This is seen also in Fig. 9 *h*. $\times 80,000$.

in their morphology depending upon whether the membrane is found in the stacked or in the unstacked configuration. Thus, we can distinguish Bs and Cs faces in stacked regions of the chloroplast and Bu and Cu faces in unstacked regions; the two types of B faces are continuous with one another, with no clear steps or ridges between them, and the two types of C faces are similarly

continuous. The relationships between fracture planes, exposed membrane faces, and particle configurations on these faces are illustrated schematically in Fig. 23, and our visualization of the organization of chloroplast thylakoids is given, again schematically, in Fig. 24.

To support our identification of the membrane faces it is important to demonstrate that Bs and

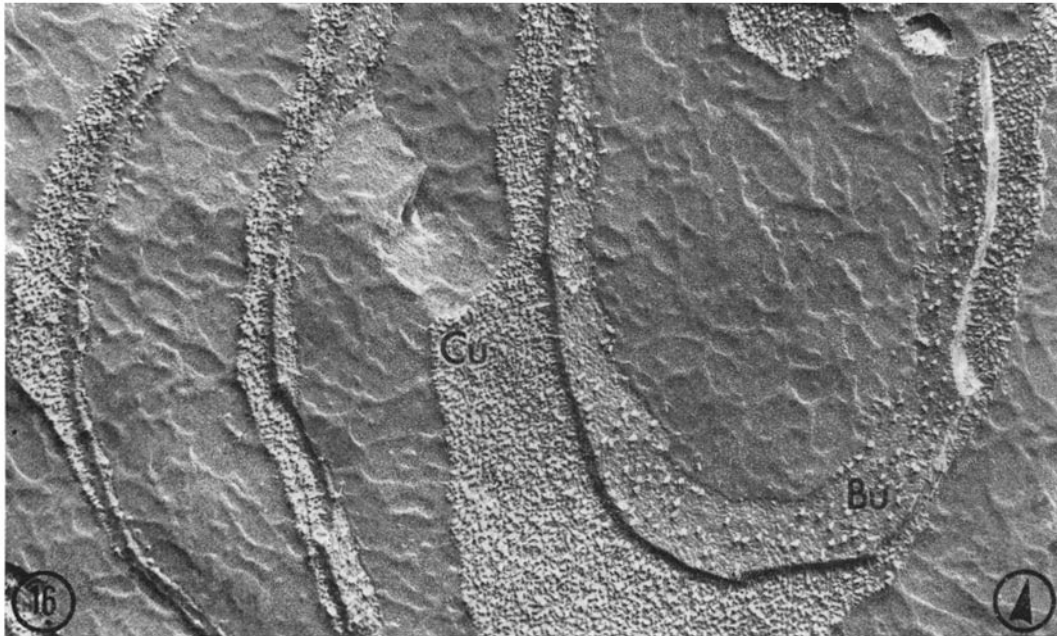


FIGURE 16 Freeze-etched, isolated chloroplast membranes from mixotrophically grown *ac-5* cells, suspended in 27% glycerol in high-salt buffer. The thylakoids are unstacked and only two different type of faces, *Bu* and *Cu*, can be distinguished. Note the higher number of particles on the *Bu* faces of these unstacked thylakoids compared with the *Bu* faces of wild-type thylakoids (Fig. 3). $\times 60,000$.

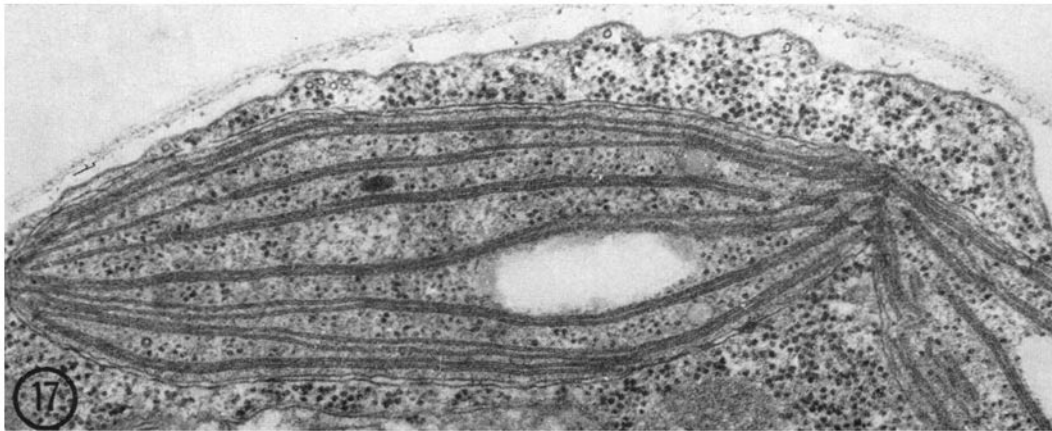


FIGURE 17 Portion of an *ac-31* cell grown mixotrophically. Single thylakoids tend to lie in closely associated pairs, but stacking does not occur between them. $\times 48,000$.

Cs faces are complementary, and also that Bu and Cu faces are complementary. Comparing first the Bs and Cs faces, it is seen that the Bs face possesses a smooth background matrix marked with numerous small depressions, each 20–80 Å in

diameter;³ the matrix also carries evenly distributed

³ As small holes and depressions are partly or completely filled with replicating material during the replication process, their apparent size is usually at

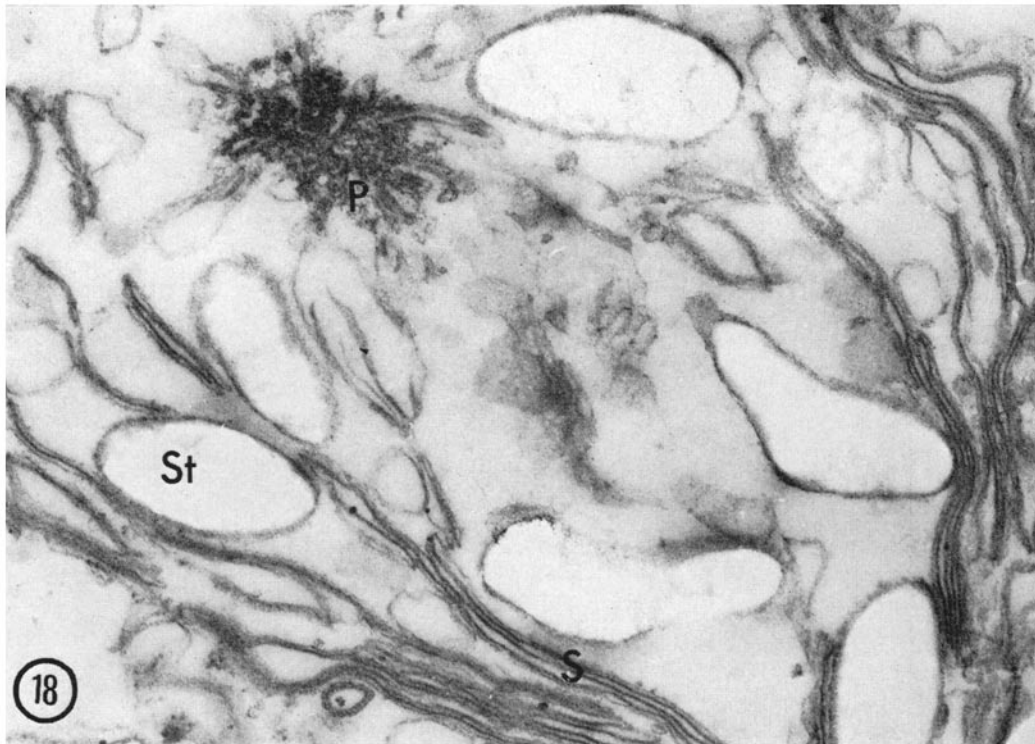


FIGURE 18 Isolated chloroplast membranes from *ac-31* cells, glutaraldehyde-fixed in 27% glycerol in high-salt buffer. Fusion of thylakoids has occurred in vitro, resulting in stacks (*S*) of from 2 to 5 thylakoids. A pyrenoid (*P*) and starch (*St*) are also seen. $\times 48,000$.

particles, a considerable proportion of which are 160 ± 10 A in diameter (Figs. 3, 5, and 9 *a*). The Cs face is rough and irregular, partly because it contains numerous large holes; these are located between small particles having diameters of 30–100 A each (Figs. 3, 4, 6, and 9 *c*). It thus seems reasonable to suppose that the large particles on the Bs faces are torn out of the large holes on the Cs faces, and that the small depressions on the Bs faces are produced by the breaking away of the small particles observed on the Cs faces.

A similar "fit" can be visualized between the Bu and Cu faces. The Bu faces exhibit a background matrix that is marked by large numbers of depressions 50–120 A in diameter; these faces also carry a sparse population of variously sized particles (Figs. 3, 7, and 9 *b*). The Cu faces are richly populated with particles (Fig. 8). Where the matrix

least 20–30 A smaller than their real size. In thick replicas, holes with diameters of less than 60 A are often completely lost.

between the Cu particles can be observed, it appears smooth except for some occasional large holes. Thus Bu and Cu faces also seem to exhibit a reciprocal distribution of particles and holes. This point can be established unambiguously only by double-replica experiments (55), but our observations are certainly consistent with the notion that we are viewing complementary faces of two differentiated, but continuous, regions of the chloroplast membrane.

In studies by other investigators, the chloroplast membrane of green plants has been considered to possess only two types of inner faces (3, 9); these correspond to our Cu and Bs faces. Our Cs face has been termed the A surface by Branton and Park (9), who propose that it corresponds to the outer surface of a stacked chloroplast membrane. We cannot agree with this interpretation, one reason being that the Cs face is coplanar with the Cu face (Fig. 4) which is, by Branton and Park's interpretation as well as our own, an internal membrane face. Our Bu face is found in replicas



FIGURE 19 Isolated chloroplast membranes from *ac-31* cells, glutaraldehyde-fixed in 27% glycerol in Tricine buffer. Single discs remain far apart without any regions of contact. The membranes appear fragmented and stain poorly, presumably because of the glutaraldehyde fixation in Tricine. $\times 48,000$.

from higher-plant chloroplasts (3, 9); other investigators have referred to it as a face which the large (Bs) particles have been pulled away during the fracturing process (see Fig. 5 of reference 9).

The Particles Found on Chloroplast Membrane Faces

The diameter of particles in freeze-etched replicas is determined, in part, by a subjective decision as to how much of the shadow of a given particle should be included in the measurement; it is also affected by such factors as pretreatment of the specimen, plastic deformation, water vapor sublimation and condensation, the thickness of the platinum/carbon shadowing layer, and the thickness of the carbon replicating layer. Because of these many sources of variability, we feel that a histogram of all the particle sizes found on a given membrane face (Fig. 9) is more meaningful than an average or a maximum particle size value.

As we have pointed out earlier, the particles on the Bs face are the most distinctive feature of freeze-etched chloroplast membranes. Mühlethaler et al. (39) report that this face carries a large, 120 A particle, whereas Branton and Park (9) report that the same face carries a large, 175 A particle. Our histograms (Fig. 9) indicate that this face contains a broad spectrum of particle sizes, with three maxima at 105 ± 10 A, 130 ± 10 A, and 160 ± 10 A. The average size of all of these particles lies in the 120–130 A range, which perhaps corresponds to the 120 A value of Mühlethaler et al. (39). The largest sized particles in our replicas presumably correspond to the 175 A particles of Branton and Park (9).

Our observations would indicate that to characterize chloroplast membrane faces by a single particle size is at present an oversimplification. On the other hand, we are in no position to state how many different types of particles a given face carries. It is certainly possible that some, and

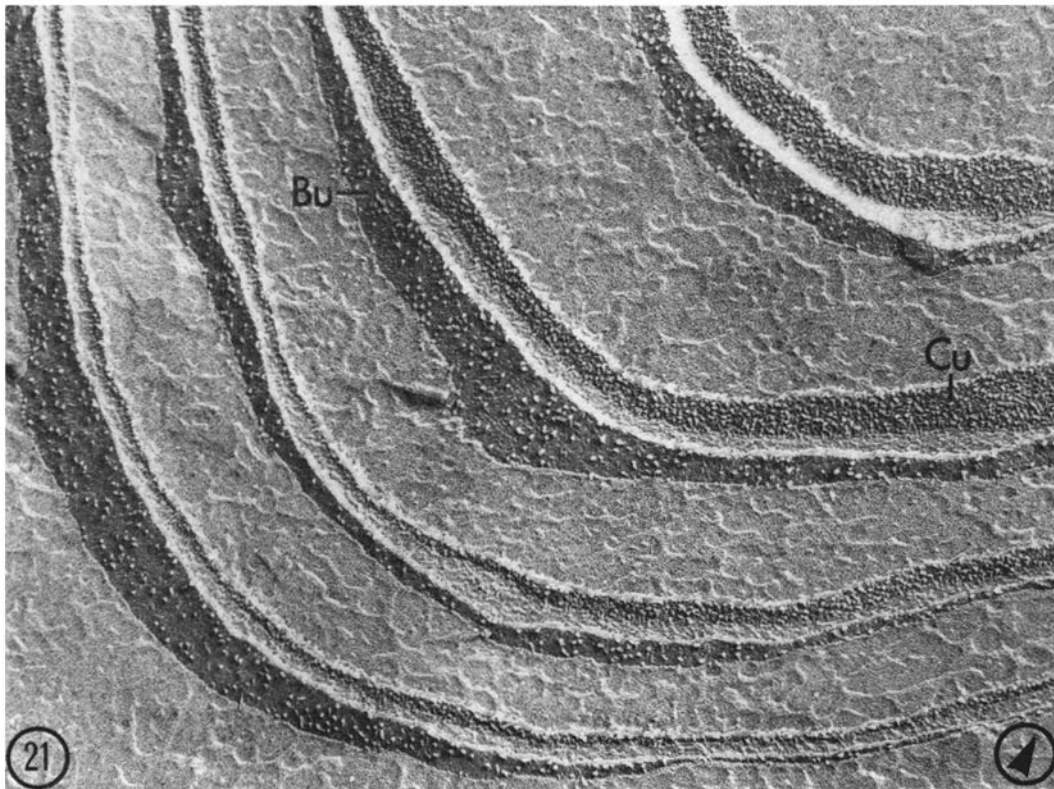
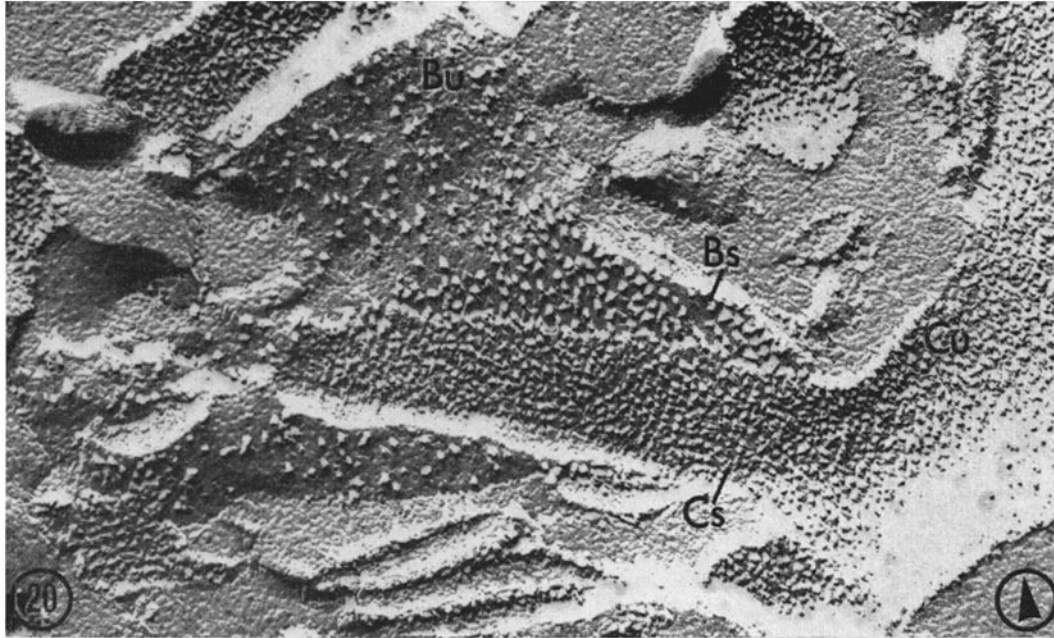


FIGURE 20 Freeze-etched isolated chloroplast membranes from *ac-31* cells, suspended in 27% glycerol in high-salt buffer. The membranes have stacked *in vitro* and exhibit faces that are indistinguishable in appearance and in their particle populations from the corresponding faces of wild-type cells (compare with Fig. 3, and see also Fig. 9, *i* and *j*). Circled triangles in lower right corner of Figs. 20-22 indicate angles of shadowing. $\times 80,000$.

FIGURE 21 Freeze-etched, isolated chloroplast membranes from *ac-31* cells, suspended in 27% glycerol in low-salt Tricine buffer. The completely unstacked thylakoids reveal only two types of membrane faces, *Bu* and *Cu*. $\times 50,000$.

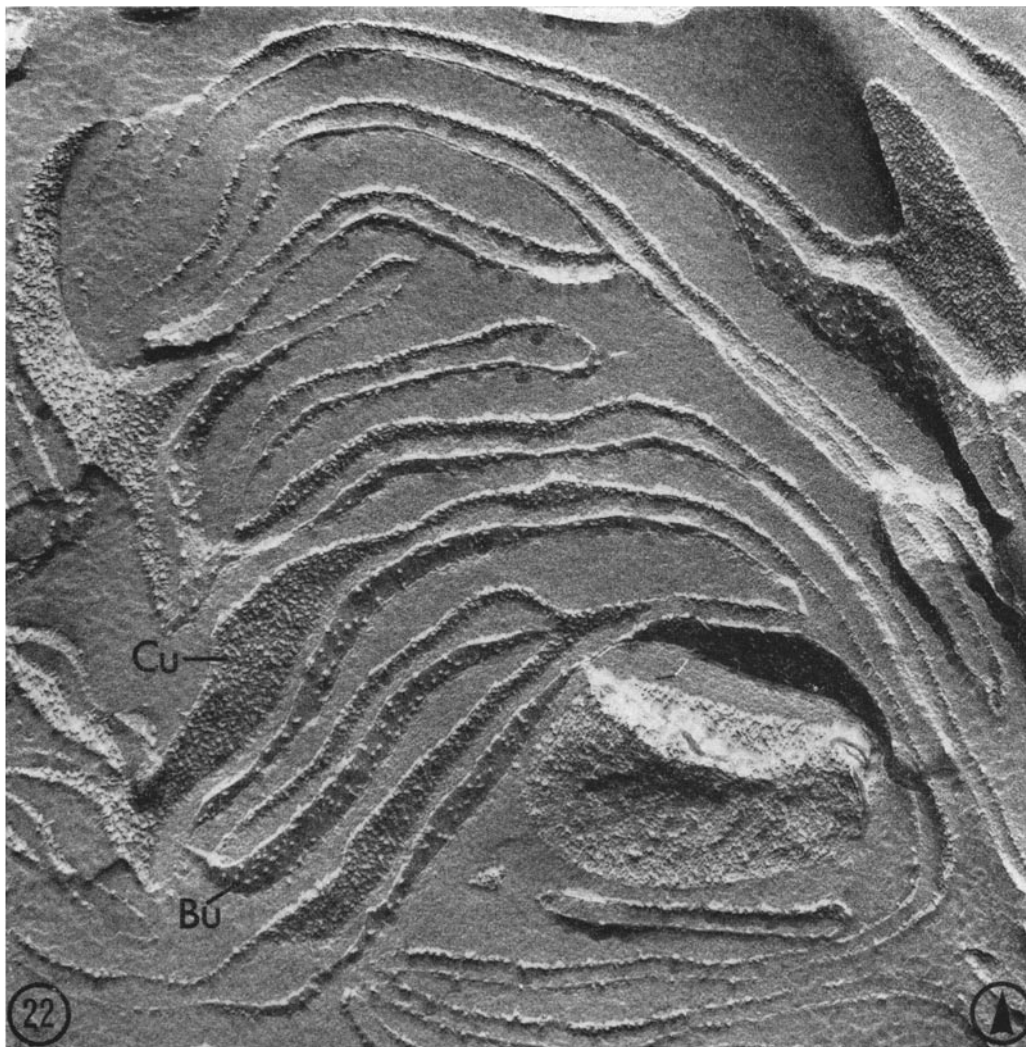


FIGURE 22 Freeze-etched, isolated chloroplast membranes from wild-type cells, isolated in high-salt buffer and then washed extensively in low-salt Tricine buffer before the addition of 27% glycerol. The thylakoid membranes have been completely unstacked by the low-salt washings. Only two types of faces, *Bu* and *Cu*, can be found in regions where the thylakoids are well separated. $\times 60,000$.

perhaps many, of the smaller-sized particles in a replica represent damaged fragments or dissociated subunits of larger-sized particles. For example, the fairly regular spacing of particles on the *Bs* face (Figs. 3, 5, 6) would suggest a more uniform population of particles on this face than the size-distribution data reveal. The peaks in the size-distribution histogram for a *Bs* face (Fig. 9 *a*), therefore, might reflect subunits or fragments of the $160 \pm 10 \text{ \AA}$ particle rather than unique species of particles. If this is the case, then a change

in the conditions under which the membrane is processed might greatly increase (or decrease) the number of $160 \pm 10 \text{ \AA}$ particles observed; in other words, the fact that our *Bs* histograms are quite uniform from one preparation to the next (compare Figs. 9 *a*, *e*, and *i*) may simply reflect the uniformity of our preparative procedures.

A related kind of problem arises when comparing one membrane face with another. The *Bu* face, for example, carries a small number of particles (some 8% of the total) with diameters of $160 \pm 10 \text{ \AA}$, but we are unable to determine whether these are

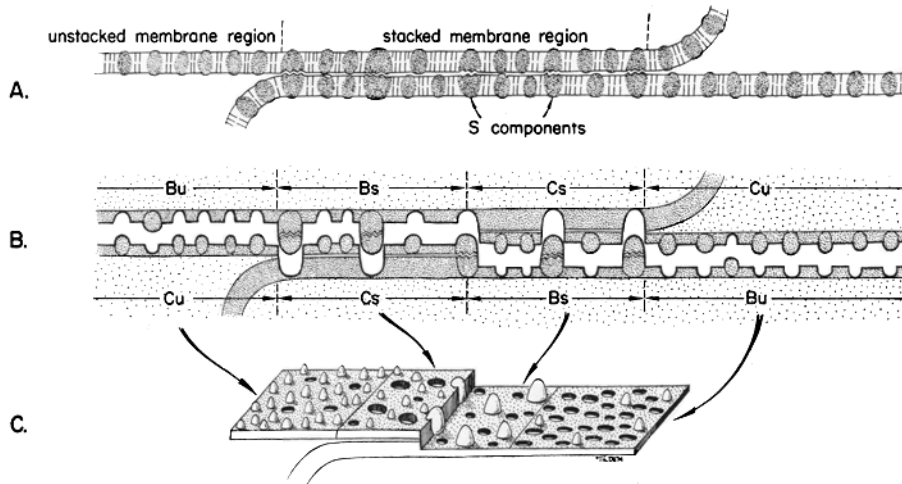


FIGURE 23 *A*, Schematic drawing showing chloroplast membranes of two adjacent thylakoids that are stacked in the region defined by the two dashed lines. Elements seen in freeze-etch replicas are depicted within the membranes as stippled areas. Regions of lipid bilayer are also indicated. The *S*-components represent the suggested membrane regions that participate in the stacking process. The jagged lines indicate regions where the *S* components of adjacent membranes make contact. Fig. 23 *B*, Diagram indicating how the hypothetical chloroplast membrane of Fig. 23 *A* could give rise to the cleavage faces observed in freeze-etch replicas. Note the complementary features of *Bu* and *Cu*, and of *Bs* and *Cs* faces. Fig. 23 *C*, Artist's view of the faces produced during the cleaving process indicated in Fig. 23 *B*.

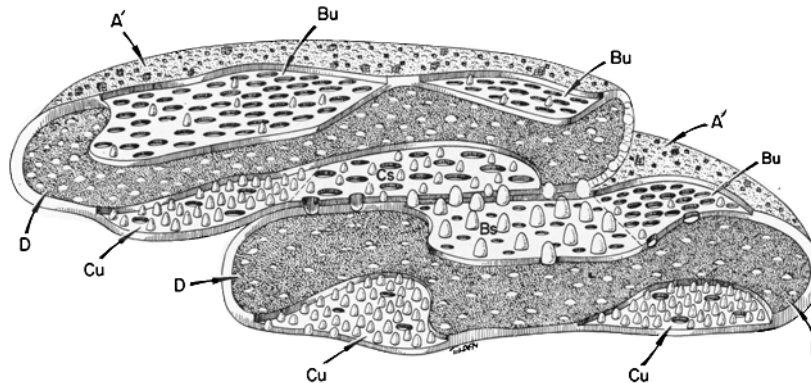


FIGURE 24 A schematic representation of two chloroplast thylakoids showing stacked and unstacked membrane regions. As revealed by freeze-etching, faces *Bu* and *Cu* and faces *Bs* and *Cs* exhibit split inner membrane faces that have complementary features. *Bu* and *Cu* faces are observed where the membranes are unstacked and *Bs* and *Cs* faces are observed where the membranes are stacked. The large particles on the face *Bs* are apparently formed in response to the stacking process. We have adopted the nomenclature and the features of the membrane surfaces marked *A'* and *D* from information provided by Arntzen et al. (3), Howell and Moudrianakis (28), and Park and Pfeiffer (42, 43).

identical with, related to, or different from the similarly sized particles on *Bs* faces. Until some procedures are devised for characterizing the biochemical nature of particles observed in freeze-etch replicas, such problems will probably remain unresolved.

Chloroplast Membrane Stacking and Particle Formation

Unstacked *C. reinhardtii* chloroplast membranes possess only *Bu* and *Cu* faces. This is true of naturally occurring, unstacked, wild-type membranes, as well as wild-type membranes whose

stacked configuration has been experimentally disrupted by washing with low-salt Tricine; it is also true of mutant membranes that have lost the ability to stack. Conversely, the Bs and Cs faces are found only where membranes are stacked, whether this stacking has occurred within the cell or in vitro. Thus, we conclude that the Bs and Cs faces are formed as a consequence of the stacking process, and we propose that their formation reflects some conformational change that occurs within the membrane at the time of stacking. It seems reasonable to propose that, as fusion occurs between the surfaces of two membranes, unique kinds of stresses are produced within the membrane interior and that these stresses could lead to associations between membrane components that appear as particles in freeze-etch replicas.

The most conspicuous difference between the faces of stacked and unstacked chloroplast membranes is the dramatic increase of 160 ± 10 A particles on the Bs face and their fairly orderly array on that face. Thus, these particles may reflect regions of the membrane that are directly involved in stacking, which would imply that fusion takes place at discrete, closely spaced, and well-ordered intervals along the membrane surface rather than over the entire membrane surface. We have indicated this type of membrane-membrane interaction in our diagrams (Figs. 22 and 23). We stress that all of our proposals as to the morphological nature of chloroplast membrane stacking are speculative.

The arrays of particles that characterize stacked chloroplast membranes are specific to the chloroplast system. Thus the membrane interiors of fused myelin membranes are apparently particle free (7), and interactions between plasma membranes in junctional complexes produce freeze-etch patterns very different from those seen in chloroplast membranes (20, 49). Of particular interest is the fact that the chloroplast membranes of the mixotrophically grown *ac-5* strain of *C. reinhardi* are unable to stack in vivo; in vitro, the isolated membranes occasionally make contact, but this contact does not result in the formation of Bs and Cs particle configurations. Thus, true membrane stacking involves more than a simple apposition of chloroplast membrane surfaces; elements within the membrane interior apparently become involved in the interaction as well, and this type of interaction is evidently prevented by the *ac-5* mutation.

Stacking of photosynthetic membranes occurs in many (4, 11, 12, 15, 17, 18, 25, 27, 46, 52), but not all (10, 11, 26), photosynthetic bacteria, in all algae but the blue-green, red, and brown classes (see reference 21), and in all higher plants with the exception of those chloroplasts located in the bundle-sheath cells of certain grasses (34). We have surveyed the literature to determine the size and distribution of particles in freeze-cleaved or freeze-etched membranes from various photosynthetic organisms other than higher plants (5, 6, 14, 18, 24, 27, 48), but extensive variability in the quality of the preparations makes it very difficult to compare one micrograph with another. We have yet to encounter a replica in which distinctive arrays of large particles are unmistakably present on photosynthetic membrane faces that are not stacked. On the other hand, it is certainly possible that certain photosynthetic organisms may have evolved ways of bringing about membrane stacking that do not involve the formation of 160 ± 10 A particles. Thus, our proposal that stacking creates arrays of these particles applies, at least at present, only to the chloroplast membranes of green algae and higher plants.

The widespread occurrence of stacking among photosynthetic organisms strongly suggests that such a membrane configuration plays some role in the photosynthetic process. No "function" for membrane stacking in photosynthesis has as yet been experimentally demonstrated, however, although several possible functions have been suggested (21). The problem is presently under investigation in the laboratory of R. P. Levine.

The 160 ± 10 A Particle and Photosynthesis

The numbers of large 160 ± 10 A particles present within chloroplast membranes are greatly enhanced as the membranes change from an unstacked to a stacked configuration, but this increase is not accompanied by any notable change in the ability of chloroplast membranes to perform photosynthetic reactions at high light intensities. Thus, *ac-5* membranes carry out PS I- and PS II-mediated reactions equally well whether stacked or unstacked (Table I), and Izawa and Good showed several years ago (29) that isolated spinach chloroplast membranes that are unstacked by low-salt Tricine are capable of substantial rates of ferricyanide photoreduction. Similarly, we are unable to relate the numbers of large particles in

a membrane to the amount of chlorophyll present in the membrane.

Park and coworkers refer to the large particle as a "quantaosome" or "quantaosome core" (42, 43), and models of photosynthesis based on quantaosome arrays have been proposed (33, 57). Recently, Arntzen et al. (3) have disrupted spinach chloroplast membranes and have obtained fractions enriched for both PS II activity and for what we have termed Bs membrane faces; they therefore suggest that the large particle of the Bs face is associated with PS II. Similarly, a membrane fraction carrying only the smaller particles found on what we term a Cu face was capable of performing only PS I-mediated reactions; thus, these investigators suggest that the smaller particles that they identify in their preparations be termed "PS I markers." Since the PS II-containing fractions are composed primarily of stacked membranes (see Fig. 6 of reference 3), and since the PS I-containing fractions are composed solely of unstacked membranes (see Fig. 10 of reference 3), we feel that the results of Arntzen et al. could be equally well interpreted by proposing that the large particle "marks" stacked membranes in their preparations, and that the smaller particle "marks" unstacked membranes in their preparations. In other words, we do not feel that any direct association between freeze-etch particles and photosynthesis has yet been demonstrated. We would like to take this opportunity to suggest that the tendency to regard the photosynthetic membrane as possessing quantized morphological structures that correspond to functional photosynthetic units be suspended until there is more conclusive evidence that such structures exist.

Factors Controlling Chloroplast Membrane Stacking in C. reinhardtii

Chloroplast membrane stacking in *C. reinhardtii* is conditioned, first, by the ionic environment, as has been pointed out previously for higher plants (29, 30). Thus, we are able to unstack chloroplast membranes by washing them with low-salt Tricine buffer, an observation that leads us to question the concept of stacking as a hydrophobic interaction (9). We assume that the ionic interactions required for stacking take place between membrane surfaces, and perhaps at specific sites along the membrane as we have suggested earlier in the Discussion.

Chloroplast membrane stacking in *C. reinhardtii* is also controlled by at least two Mendelian genes. The *ac-5* and *ac-31* loci have been mapped to two separate linkage groups of the *C. reinhardtii* genome (35), and mutations at these loci lead to quite distinct phenotypes. Chloroplast membrane stacking can occur in *ac-5* cells that are grown phototrophically, but it does not occur in mixotrophically grown cells,⁴ nor can it be induced if the mixotrophic membranes are isolated in a high-salt buffer. The *ac-31* strain, on the other hand, does not exhibit stacked chloroplast membranes in whole cells under either growth condition; however, when the membranes are isolated in a high-salt buffer, they are capable of true stacking *in vitro*, and they can be kept apart *in vitro* only by isolating them in a low-salt buffer.

Two interpretations can be offered for the *ac-31* mutation. The mutation might result in some alteration of the chloroplast stroma such that the proper ionic environment for membrane stacking is not provided by the intact cells. Alternatively, the mutation might produce an alteration in some membrane component that participates in membrane stacking. As a result, the normal ionic milieu of the stroma might no longer be adequate, or appropriate, for the stacking process, whereas the high-salt buffer in the chloroplast isolation medium would be appropriate.

The buffer used to isolate stacked chloroplast membranes contains high concentrations (0.02 M) of magnesium. Since divalent cations have been frequently found to be involved in the adhesive properties of biological components, we are presently exploring the possibility that magnesium specifically mediates chloroplast membrane stacking.

It should be pointed out that Park and Pfeiffer (43) report the presence of Bs faces in spinach membranes that have been isolated in 0.02 M Tricine. They state that A faces are rare in their preparations, implying that stacking is limited, but they do not establish by thin sectioning that their membrane fragments are totally unstacked.

⁴ Such a differential expressivity is known for two other gene mutations in *C. reinhardtii*: the phenotypes of the *ac-20* (23, 36, 54) and the F-54 (Chua and Sato, unpublished) strains are also distinctly different under phototrophic and mixotrophic growth conditions, a phenomenon that cannot be explained at the present time.

Anderson and Vernon (1), repeating the Tricine procedures of Izawa and Good (29), obtain only partially unstacked spinach preparations, and Kahn and von Wettstein (32) observe stacking in spinach chloroplast fragments that are suspended in distilled water. We find that, in *C. reinhardi*, extensive washing in five changes of low-salt Tricine buffer still leaves some intact stacks in wild-type membrane preparations. Taken together, these results indicate that rigorous criteria must be used to determine that a given preparation is unstacked, particularly when one is attempting to pull apart membranes that are initially tightly fused.

The Differentiation of the Chloroplast Membrane

Izawa and Good (29) have reported that when they add salts back to isolated spinach chloroplasts that have been unstacked in a low-salt Tricine buffer, normal looking grana often assemble in vitro. We have made a similar observation with *C. reinhardi*: when *ac-31* membranes are provided with a high-salt environment in vitro, the normal *C. reinhardi* pattern of membrane association occurs, and not some massive, nonspecific aggregation of membranes. Since this pattern is formed by isolated, washed membranes, we conclude that a specific basis for the patterning is built into the structure of the membranes themselves. In other words, it appears that certain regions of a chloroplast membrane are capable of stacking, and that adjacent regions are not. We have not been able to detect any obvious manifestation of this patterning in freeze-etch replicas; thus the Bu and Cu faces of wild-type and *ac-31* membranes that have been isolated in low-salt Tricine do not reveal any patterning of particle sizes or densities that we might relate to potential regions of stacking and unstacking. This does not seem surprising, however, for we presume that if a morphological manifestation of chloroplast membrane differentiation exists at all, it should be found on the membrane surface and not in the membrane interior.

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Addendum. After this manuscript had been submitted for publication, two articles that we had not previously seen were brought to our attention: Remy, R. 1969. Etude de la structure des lamelles chloroplastiques de *Vicia faba* L. par la technique du cryodécapage. *C. R. Acad. Sci.* 268:3057, and Phung Nhu Hung, S., A. Lacourly, and C. Sarda. 1970. Etude de l'évolution en chloroplastes des plantes etiolées d'orge. I. Structure examinée par cryodécapage. *Z. Pflanzenphysiol.* 62:1. These authors have observed that membrane faces identical to our Bs faces occur only in stacked regions of bean and barley chloroplasts, respectively. We acknowledge the priority of these authors in making this observation. It appears that, at least on this point, our interpretation of chloroplast membrane structure applies not only to *Chlamydomonas* but to higher plants as well.

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